

The University of Chicago

**A COMPARATIVE STUDY OF CULTURAL
AND IMMUNOLOGICAL METHODS OF
DIAGNOSING INFECTION WITH END-
AMOEB A HISTOLYTICA**

A PART OF THE DISSERTATION SUB-
MITTED TO THE GRADUATE FACULTY
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND
BACTERIOLOGY

By
BERTHA KAPLAN SPECTOR

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A COMPARATIVE STUDY OF CULTURAL AND IMMUNOLOGICAL METHODS OF DIAGNOSING INFECTIONS WITH *ENDAMOEBIA HISTOLYTICA**

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In this study the attempt has been made to develop a more efficient method for diagnosing infections with *Endamoeba histolytica* than is now in practice. It has involved the perfection of a culture medium containing as few non-human proteins as possible, the preparation of suitable antigens, and a comparative study of various immunological techniques.

Cutler,¹ in 1918, was probably the first to grow *E. histolytica*; one of his media was a liquid egg-and-water medium. Among the numerous investigators who since that time have reported media permitting the growth of this organism are, Boeck and Drbohlav,² Drbohlav,³ Brumpt,⁴ Tanashe and Chiba,⁵ Dobell and Laidlaw.⁶ The media, however, most widely used are those of Boeck and Drbohlav² (egg slants covered with 1 per cent egg albumin in Locke's solution, or with

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¹ Cutler, D. W.: Jour. Path., 1918-19, 22: 22-27.

² Boeck, W. C., and Drbohlav, J.: Amer. Jour. Hyg., 1925, 5: 371-407.

³ Drbohlav, J. J.: Ann. de Par., 1925-26, 3-4: 349-366.

⁴ Brumpt, E.: Bull. Medical, 1926, 40: 105-109.

⁵ Tanashe and Chiba, quoted by Cleveland and Collier: Amer. Jour. Hyg., 1930, 12: 606-613.

⁶ Dobell, C., and Laidlaw, P. P.: Parasitol., 1926, 18: 283-318.

1 part of inactivated human, horse or rabbit serum to 8 parts of Locke's or Ringer's solution); of Craig⁷ (solid egg slants covered with inactivated human serum Ringer's solution or with human serum diluted 1:7 with 0.85 per cent NaCl); of Dobell and Laidlaw⁸ (inspissated horse serum slants covered with a sterile mixture of fresh horse serum and Ringer's or Locke's solution or egg white with Locke's or Ringer's solution, sterilized by filtering through a Seitz filter, and rice starch added); and of Cleveland and Sanders⁸ (liver infusion agar slants prepared as follows: 30 grams liver infusion agar, 3 grams Na₂HPO₄, 0.5 cc. washed red blood cells, and 1 liter distilled water; overlaid with sterile horse serum diluted with 6 parts NaCl to which a 5-mm. loop of sterile rice flour is added just before inoculations). In my own work I have found Cleveland and Sander's media the most convenient for practical diagnosis.

Among the first to apply serological methods to the diagnosis of *E. histolytica* infections was Izar,⁹ who in 1914 reported a series of complement fixation tests with an antigen consisting of an aqueous extract of feces and liver-abscess pus from an infected cat. Hage¹⁰ used the same antigen as Izar, but found it unsatisfactory. Scalas,¹¹ on the other hand, confirmed Izar's work by using an antigen consisting of an aqueous extract of mucous flakes from amoebic stools. Craig,¹² who made the most extensive series of complement fixation tests, used alcoholic extracts of sediment from *E. histolytica* cultures. Precipitin tests of serums from kittens were performed by Wagener,¹³ with an antigen consisting of extracts of scrapings of the ulcerated intestines of cats. Scalas,¹⁴ in carrying out a series of intradermal tests, used an antigen prepared from mucus and mucous flakes of fresh stools from amoebic dysentery patients.

⁷ Craig, C. F.: Jour. Trop. Med., 1926, 6: 461-464; Amer. Jour. Trop. Med., 1926, 6: 333-339.

⁸ Cleveland, L. R., and Sanders, E. P., Science, 1930, 72: 149-151; Arch. f. Protist., 1930, Bd. 70.

⁹ Izar, G.: Arch. f. Schiffs- u. Tropenhyg., 1914, 18: 36.

¹⁰ Hage: Deutsch. med. Wchnschr., 1920, 46: 682-684.

¹¹ Scalas, L.: Riforma med., 1921, 37: 103-104.

¹² Craig, C. F.: Amer. Jour. Trop. Med., 1927, 7: 225-240; Nat. Acad. Sci., 1928, 14: 520-526; Amer. Jour. Trop. Med., 1928, 8: 29-37.

¹³ Wagener, E. H.: Univ. California Publications in Zool., 1923-25, 26: 15-20.

¹⁴ Scalas, L.: Riforma med., 1923, 39: 967, 969.

While the results of Craig with complement fixation, of Wagener with the precipitin test and of Scalas with intradermal tests are encouraging, they indicate that immunological means of diagnosing infections with *E. histolytica* cannot always be relied upon. The difficulty lies in the following: (1) the preparation of an adequate antigen, (2) the correlation of a sufficiently large number of patients with the examinations of stools and histories to make the results significant, (3) proper controls and unbiased reading of results. A careful study of Craig's complement fixation tests discloses that occasionally persons who are free from amoebic infections give positive complement fixations, while other persons with amoebic infections fail to give positive complement fixation reactions. Although the number of such cases is comparatively small the fact that the total number of cases of amoebic dysentery is also small makes such discrepancies significant. Furthermore, those cases which are most difficult to diagnose microscopically and culturally are the ones which are most frequently missed immunologically.

MATERIALS AND METHODS

Culture media. Solid slants consisting of three parts of inactivated Wassermann negative human serum and one part of Ringer's solution or 0.85 per cent NaCl solution proved more satisfactory than any of the other solid media tried (human-blood chocolate agar, Loeffler's medium, dextrose agar, starch agar, Boeck and Drbohlav's medium, Ringer's-solution egg medium). These slants were overlaid with a sterile mixture of one part of inactivated Wassermann negative human serum and six parts of Ringer's solution or 0.85 per cent NaCl solution (sterilized by filtering through a Berkefeld filter). Before subcultures were made, a little sterile rice flour or rice starch was added. On such slants luxuriant growth of *E. histolytica* was produced after 24 or (usually) 48 hours' incubation. As this medium was devoid of non-human protein the antigen prepared from the heavy growth of organisms obtained therefrom was preferable to antigens prepared from amoebic stools, liver abscesses, scrapings of cat intestines, etc., all of which contain much foreign protein. For practical diagnostic purposes, however, liver infusion agar and dehydrated Loeffler's medium, recommended by Cleveland and Sanders,⁸ whose paper was

published a year after my work was started, have been found advantageous, in that they can be purchased and prepared readily.

Two other liquid media to overlay the slants were tested, but did not work so well as the one described above: (1) seven parts of Locke's solution and one part of inactivated Wassermann negative human serum; (2) seven parts of Ringer's solution and one part of inactivated Wassermann negative human serum. These solutions were sterilized by filtering through a Berkefeld filter.

Antigens for complement fixation. Two types were prepared: (1) One part of sediment from 24-to-48-hour cultures of *E. histolytica*, from which as many bacteria as possible had been washed away, was extracted in 7.5 parts of absolute alcohol for 15 days at 37.5°C. During this time the mixture was shaken frequently by hand, as well as in a rotating apparatus for 48 hours. (2) A 1 per cent absolute-alcohol extract of the antigen just described, after it had been dried. The first of these antigens, with its respective control, gave better and more clear-cut results and was used in the specific complement fixation tests reported in this paper.

The antigen controls consisted of similar extracts of the sediment of 24-to-48-hour cultures of the aerobic and anaerobic bacteria grown in association with the amoebae. The bacterial cultures were grown in exactly the same number of tubes, in the same medium and for the same period of time as the amoebae. A third antigen control consisted of an alcoholic (absolute alcohol) extract of rice starch sediment from the same number of tubes, prepared and incubated in exactly the same way as the cultures, but uninoculated.

Upon completion of extraction each antigen and antigen control was centrifuged in large chemically clean sterile centrifuge tubes until the supernatant fluid was clear. The supernatant fluid was then filtered through sterile funnels into chemically clean sterile glass-stoppered bottles, and titrated.

The antigenic dose (one-half of the anticomplementary dose) of the test antigen was 0.3 cc. of a 1:2 dilution, and of the bacterial control antigen was 0.1 cc. of a 1:2 dilution.

Antigens for skin tests. Three methods were used: The first antigen consisted of equal parts of washed sediment of seven different strains of *E. histolytica* and Coca's solution, the mixture being heated for one

hour at 60°C. on several successive days and incubated at 37.5°C. between each heating. Since sterility tests showed that *B. welchii* spores could not be killed in such a concentrated mixture or dilutions of it, the extract was filtered through a Seitz filter. This first antigen proved the best and was used in performing the tests.

The second skin-test antigen consisted of a 1 per cent extract in Coca's solution of the washed and dried sediment of cultures of *E. histolytica* showing heavy growth. The sediment was dried in a desiccator, ground in a sterile mortar, extracted in the ice-box for 2 days and centrifuged. The supernatant fluid was then filtered through a Seitz filter. The third antigen consisted of a 1 per cent extract in Coca's solution of a dried antigen prepared by extracting the washed sediment of cultures of *E. histolytica* with seven and a half parts of absolute alcohol in the incubator for 15 days, shaking frequently by hand, and then shaking in a rotating apparatus for 48 hours; it was filtered through a Seitz filter. This last preparation had no antigenic value.

The antigen controls consisted of exactly similar extractions of the aerobic and anaerobic bacteria grown in association with the amoebae, in exactly the same medium for the same period of time and in the same number of tubes, and, like the antigens, filtered through a Seitz filter. The filter was washed and sterilized between each filtration.

Sterility tests on both antigens and controls were made aerobically and anaerobically for a week to make certain that the extracts were sterile. The antigen and antigen controls were then put in small chemically clean sterile vials.

Antigens for precipitin tests. Three test antigens were prepared exactly as were those for the skin tests, except that they were filtered through hard filter paper instead of through a Seitz filter. The antigen prepared by the first method (above), which had proved best for the skin test, was likewise best for the precipitin test and was used in carrying out the tests here reported. Ring tests were made by overlaying the serums with undiluted antigen and 1:2, 1:4, 1:8 dilutions. Controls were performed with the corresponding antigen control and with saline. The saline controls were consistently negative but the bacterial antigen controls were quite frequently positive. Readings were made after 15, 30, 45 and 60 minutes at room temperature; then

after incubating in a water bath at 37.5°C. for one hour; and finally after remaining in the ice-box over night. The results after one hour at room temperature were best and are the ones recorded.

EXAMINATIONS AND TESTS

Fecal smear and cultural methods. In the present work a total of 212 persons were examined for *E. histolytica* by both direct fecal smear and cultural methods. By the smear method 10 were found infected; by the cultural method the same 10 were found infected and 5 others. Some of the additional infections could undoubtedly have been disclosed by repeated examinations of smears. In two cases, however, a series of five stools failed to reveal any amoebae by direct smear,

TABLE 1
Correlation between infection with E. histolytica and the delayed skin reaction

E. HISTOLYTICA INFECTION	SKIN REACTIONS				TOTAL
	Tests + Controls —	Tests + Controls +	Tests — Controls —	Tests — Controls +	
Positive: dysentery.....	2				2
Positive: carrier.....		2			2
Negative.....	6	27	94	26	153
Total.....	8	29	94	26	157

although they were all positive when cultured. While the present series includes too few infected cases to give a valid statistical conclusion, it indicates that the cultural method is superior to the smear method.

Skin tests. Most of the skin tests were performed on patients at the Cook County Hospital, Chicago. At first, two skin tests with different antigens and adequate controls were made, one pair on each arm of a patient, but only the data obtained by using the best antigen are recorded in table 1. The test consisted of an intracutaneous injection of 0.1 cc. of antigen in the upper forearm and the same amount of its respective control antigen in the lower forearm. Readings were made after 20 minutes, 24, 48 and 72 hours. At the same time blood was drawn for specific complement fixation tests and stools

were collected for examination for parasites, cysts and ova, and for cultures for *E. histolytica*.

Very few persons showed any reaction after 20 minutes (immediate reaction), but some showed local reactions after 24 hours (delayed reaction). The latter ranged from a slight reddening to quite severe swelling, hemorrhage and erythema which subsided within 48 to 72 hours. In no case were there any systemic disturbances. In one patient suffering from an acute amoebic dysentery, the test antigen produced a severe reaction, consisting of local hemorrhage, erythema, edema and swelling, which lasted for 48 hours; the control antigen was definitely negative. At the same time a patient with bacillary dysentery gave clear-cut negative results. This seemed very encouraging, but the more cases tested, the more varied and less consistent did the results become. A case of amoebic colitis showed the same degree and type of reaction with the control as with the test antigen. Some infected patients gave clear-cut negative results with both the test and the control antigens, while others showed various degrees of reactions with the antigens. In such cases the control antigen seemed to have produced stronger reactions than the test antigen, regardless of the type of infection.

The results of the tests on 157 patients may be found in table 1. The fact that all four of the patients with amoebiasis gave positive reactions is very interesting, but is somewhat mitigated by the fact that in the two patients with chronic symptoms the controls were positive as well. The question of a satisfactory control in carrying out skin tests is of the utmost importance since it has been found that individuals may not only be positive to the diluent of the testing fluid but to proteins in general (Taliaferro).¹⁵ In such an eventuality the test subject has to be considered unsatisfactory and the test ruled out.

In only two of the patients infected with *E. histolytica* were other parasites found—sprue and hookworm in one case, *Giardia lamblia* and hookworm in the other. Both these patients gave strongly positive complement fixation tests.

Among the six persons who, so far as could be ascertained, were not

¹⁵ Taliaferro, W. H.: "The Immunology of Parasitic Infections," 1929. New York and London (The Century Co.).

infected with *E. histolytica*, but who gave positive tests, three were infected with *E. coli*, one with *Endolimax nana* and *Trichuris trichiura*, and one with hookworm. Among the 27 persons in whom both the test and control antigens gave positive results, there were: one infection with *Ascaris lumbricoides*, four with *E. coli*, one with *E. coli* and *Giardia lamblia*, and one with *Trichostrongylus*. Among the 94 persons not infected with *E. histolytica* who responded negatively to the test antigen, there were nine infected with *E. coli*, one with *E. coli* and *Chilomastix mesnili*, one with *Chilomastix mesnili* alone, two with *Giardia lamblia*, two with *Trichomonas hominis*, two with *Endolimax nana*, two with *Trichuris trichiura*, two with *Strongyloides stercoralis* and one with *Oxyuris vermicularis*. Of the 26 persons who elicited positive reactions with the control but not with the test antigen, one

TABLE 2
Correlation between infection with E. histolytica and the precipitin test

E. HISTOLYTICA INFECTION	PRECIPITIN TESTS				TOTAL
	Tests + Controls —	Tests + Controls +	Tests — Controls —	Tests — Controls +	
Positive: dysentery.....	3	3	0	0	6
Positive: carrier.....	1	3	3	0	7
Negative.....	7	32	84	1	124
Total.....	11	38	87	1	137

was infected with *Trichuris trichiura* and one with *Trichuris trichiura* and *Endolimax nana*.

Precipitin tests. The data for 137 precipitin tests will be found in table 2. Since 38 of the serums reacted positively both to the control and to the specific antigen, and one serum gave a positive control and negative specific test, the precipitin test does not appear to be of any diagnostic significance so far as the technique which I have used is concerned.

Complement fixation. In carrying out the complement fixation tests both the anti-human (Jansky type I human cells) as well as the anti-sheep methods were used at first. The anti-human system was eventually discontinued because on the whole the anti-sheep method proved to be better and more convenient.

The native anti-sheep amboceptor of serums from persons, as well as from infected kittens giving negative tests, was absorbed according to Kolmer's method. This consists of adding 1 drop of washed sheep's cells per cc. of serum to the clot, placing the tube in the ice-box for 15 minutes, centrifuging and using the serum in the usual way.

The serums were inactivated at 56°C. for 30 minutes before the tests were performed. Readings were made after the final water bath incubation and after the tubes had been left in the ice-box over night. Those giving 3 and 4 plus reactions were considered positive.

TABLE 3

Correlation between infection with E. histolytica and complement fixation test, using the anti-human method

INFECTION WITH <i>E. HISTOLYTICA</i>	COMPLEMENT FIXATION TESTS		TOTAL
	Positive	Negative	
Positive.....	4	0	4
Negative.....	6	99	105
Total.....	10	99	109

TABLE 4

Correlation between infection with E. histolytica and complement fixation tests, using the anti-sheep system

<i>E. HISTOLYTICA</i> INFECTION	COMPLEMENT FIXATION TESTS		TOTAL
	Positive	Negative	
Positive: dysentery.....	6	2	8
Positive: carrier.....	2	3	5
Negative.....	14	130	144
Total.....	22	135	157

The data for 109 complement fixation tests, using the anti-human system, may be found in table 3. Of the 10 positive serums, 4 came from patients with active amoebic dysentery, who had positive stools; 6 from patients with negative stools, who were free from any symptoms of amoebic infection.

The results with the 157 serums tested by the anti-sheep method are given in table 4. The patients with dysentery from whom blood was taken for these tests were the same as those mentioned on page 123.

Among the 14 patients who were not found to be infected with *E. histolytica* but whose serums gave positive complement fixation with the anti-sheep method, the following parasitic infections were found: *Endolimax nana*, 1; *E. coli*, 2; *Strongyloides stercoralis*, 1.

Of the 135 patients who were not infected with *E. histolytica* and whose serums gave negative tests, the following parasitic infections were found: *E. coli* cysts, 17; *Endolimax nana* cysts, 3; *Strongyloides stercoralis*, 2; *Giardia lamblia* cysts, 5; *Chilomastix mesnili*, 2; *Oxyuris vermicularis*, 1; *Ascaris lumbricoides* ova, 3; hookworm ova, 4; *Trichuris trichiura* ova, 5; *Trichostrongylus* ova, 1; *Trichomonas intestinalis*, 1; *Iodamoeba bütschlii* cysts, 1.

TABLE 5
Correlation of Wassermann tests and specific complement fixation tests for *E. histolytica*
(using alcoholic antigen of the latter)

WASSERMANN	SPECIFIC COMPLEMENT FIXATION TEST		TOTAL
	Positive	Negative	
Positive.....	10	27	37
Negative.....	6	59	65
Total.....	16	86	102

Furthermore, negative reactions were obtained in a large number of surgical and medical conditions with and without parasitic infections.

RELATION OF THE WASSERMANN TEST TO THE COMPLEMENT FIXATION TEST FOR *E. HISTOLYTICA*

The question as to whether a positive complement fixation test for amoebic dysentery will affect the Wassermann reaction and *vice versa* comes up frequently. Complement fixation tests using the alcoholic extract of *E. histolytica* were done on 102 serums upon which Wassermann and Kahn tests were also performed (table 5). Although stool examinations were made for only a few of the patients whose serums are represented, the data show that a positive Wassermann reaction has no greater effect upon the outcome of the complement fixation test for *E. histolytica* than has any non-syphilitic infection.

DISCUSSION

Cultural methods for diagnosing infections with *E. histolytica* are probably superior to direct fecal examinations. The cultural methods eliminate the necessity for making stained mounted preparations because, in culture, precystic forms become active, encysted forms excyst and the vegetative organisms show the typical form, structure and rapid pseudopodial movement. *E. coli* rarely grows in the medium I have used for *E. histolytica* (preferring a more acid medium), and even when it does appear, it shows its typical form and movement which cannot be mistaken for *E. histolytica* by the trained observer.

Every specimen of stool and pus from liver or lung abscesses examined for *E. histolytica* should have cultures made, preferably on both liver-infusion agar and on dehydrated Loeffler's medium, both being overlaid with a mixture of one part of inactivated Wassermann negative human serum and six parts of NaCl, sterilized by filtering through a Berkefeld filter. The medium should be at body temperature when the inoculations are made. In making cultures of formed stools, a piece about the size of a pea should be macerated by rubbing it with the inoculating needle against the sides of the test tube during the process of inoculation. In making cultures of pus or mucoid loose stools, several loopfuls of material should be inoculated and examined after 24, 48 and 72 hours' incubation. Sterile rice flour or rice starch need not be added unless the original cultures are to be subcultured to keep the strain growing.

At present the skin and precipitin tests are inadequate for diagnostic purposes. Although the complement fixation tests are not always consistent, they offer assistance in making diagnosis especially in cases with positive histories where the complement fixation tests are positive but stools are negative. On the other hand, a positive complement fixation for *E. histolytica* does not always signify infection with *E. histolytica* and should not be interpreted as such in the absence of specific organisms, symptoms, lung or liver abscesses, or a history of infection. A negative complement fixation does not always signify the absence of such infection because of the individual variation in the production of complement-fixing antibodies and the effect of emetine upon the complement-fixing antibodies. In some patients

even one dose of emetine may change a positive to a negative complement fixation test, whereas in others even an entire series of emetine treatments will not effect the positiveness of the test.

CONCLUSIONS

1. The following medium is highly satisfactory for growing *E. histolytica*: slants prepared from three parts of inactivated Wassermann-negative human serum and one part of 0.85 per cent NaCl, overlaid with a mixture of one part of sterile inactivated Wassermann-negative human serum and six parts of either NaCl or Ringer's solution.

2. In the detection of *E. histolytica* infections, cultural methods are probably superior to direct fecal examinations.

3. A negative complement fixation test for *E. histolytica* does not always signify the absence of infection with *E. histolytica*, because individuals vary in the formation of complement-fixing antibodies and because treatment with emetine sometimes alters the reaction.

4. Likewise, a positive complement fixation test may not signify an infection with *E. histolytica*.

5. Skin and precipitin tests are, as yet, inadequate for the diagnosis of *E. histolytica* infections.

